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IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C. Part 9: Reference procedure for the measurement of catalytic concentration of alkaline phosphatase

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Scientific Division, Committee on Reference Systems of Enzymes (C-RSE)[1)]

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Abstract

This paper is the ninth in a series dealing with reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C and the certification of reference preparations. Other parts deal with: Part 1. The concept of reference procedures for the measurement of catalytic activity concentrations of enzymes; Part 2. Reference procedure for the measurement of catalytic concentration of creatine kinase; Part 3. Reference procedure for the measurement of catalytic concentration of lactate dehydrogenase; Part 4. Reference procedure for the measurement of catalytic concentration of alanine aminotransferase; Part 5. Reference procedure for the measurement of catalytic concentration of aspartate aminotransferase; Part 6. Reference procedure for the measurement of catalytic concentration of γ-glutamyltransferase; Part 7. Certification of four reference materials for the determination of enzymatic activity of γ-glutamyltransferase, lactate dehydrogenase, alanine aminotransferase and creatine kinase at 37 °C; Part 8. Reference procedure for the measurement of catalytic concentration of α-amylase. The procedure described here is derived from the previously described 30 °C IFCC reference method. Differences are tabulated and commented on in Appendix 1.

Keywords: alkaline phosphatase; IFCC reference procedure; reference intervals.

Introduction

The catalytic concentration of alkaline phosphatase (ALP) (Orthophosphoric-Monoester Phosphohydrolase, Alkaline Optimum, EC 3.1.3.1) in serum represents the activity of multiple forms of the enzyme. More than 17 isoforms of ALP are detectable by use of an isoelectric focusing technique (1). The catalytic concentration of ALP in serum of healthy adults originates from the liver and from bone in similar proportions. ALP from the small intestine contributes to approximately 10 % of total ALP in healthy individuals. If not physiologically induced by growth of bone or in pregnancy, increase of ALP in serum occurs as a consequence of disease of the liver and/or bone.

The determination of the catalytic concentration of ALP in serum depends strongly on the chosen measurement para-

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meters. Among these, the choice of the buffer is of great importance. In 1983 an expert group of the International Federation of Clinical Chemistry (IFCC) provided a proposal for a standardized measurement procedure for ALP (2, 3). The reaction principle was based on the use of 2-amino-2-methyl-1-propanol (AMP) and 4-nitrophenyl phosphate (NPP); the measurement temperature was 30 °C. Even though this procedure for ALP was never endorsed as official IFCC recommendation by the IFCC member societies, many commercial test kits for ALP currently use the AMP buffer.

This paper describes the IFCC primary reference measurement procedure for ALP, which is based on the previous IFCC work (2, 3). The change of the measurement temperature from 30 °C to 37 °C necessitated a re-evaluation of the measurement conditions. The results of this re-evaluation with very detailed definition of the measurement parameters relevant for high-level standardization are described. This paper is the ninth in a series dealing with reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C and the certification of reference preparations (4–11).

The reference measurement procedure for ALP will be of use in a reference measurement system for the certification of calibrators and control materials, thus providing the basis to allow the clinical laboratories to produce traceable measurement results and to establish definitive reference intervals (12, 13). In particular, here we provide only preliminary reference intervals for adult subjects; ALP reference intervals undergo important changes dependent on age and gender and further work is needed to obtain the full range of reference intervals.

Reaction principle

ALP catalyzes the hydrolysis of NPP, forming phosphate and free 4-nitrophenol; under alkaline conditions, 4-nitrophenol is converted to the 4-nitrophenoxide ion. AMP and H_2O are used as phosphate-acceptors:

1) 4-Nitrophenyl phosphate + H_2O $\xrightarrow{ALP}$ 4-Nitrophenoxide + Phosphate
2) 4-Nitrophenyl phosphate + AMP $\xrightarrow{ALP}$ 4-Nitrophenoxide + AMP-Phosphate

Specimens

Calibration materials, control specimens and human sera.

Measurement conditions

Concentrations in the final reaction mixture and the measurement conditions are listed in Tables 1 and 2.

Note: The compliances with the prescribed maximum allowable expanded uncertainties (95 % probability) of the values for temperature, pH, light path and wavelength are confirmed if the expanded uncertainty of the calibration (95 % probability) is equal to or smaller than the maximum allowable expanded uncertainty, **and** the result of the calibration does not differ significantly ($p \leq 0.05$) from the target value. This is valid if

$$\left| \frac{value_{target} - value_{calibration}}{\sqrt{(U_{max}^2 + U_{cal}^2)}} \right| \leq 1$$

where $value_{target}$ is the target value prescribed in the IFCC document; $value_{calibration}$ is the result determined with the calibration procedure; U_{max} is the maximum allowable expanded uncertainty prescribed in the IFCC document; and U_{cal} is the maximum expanded uncertainty of the result of the calibration procedure.

Table 1 Concentrations in the final complete reaction mixture for the measurement of ALP.

2-Amino-2-methyl-1-propanol	750 mmol/L
pH (37 °C)	10.20 ± 0.05[a]
4-Nitrophenyl phosphate	16 mmol/L
Zinc sulfate	1 mmol/L
Magnesium acetate	2 mmol/L
HEDTA	2 mmol/L
Volume fraction of sample	0.0196 (1:51)

[a]Maximum allowable expanded uncertainty (95 % probability).

Reagents

1. 2-Amino-2-methyl-1-propanol ($C_4H_{11}NO$), $M_r = 89.14$
2. 4-Nitrophenyl phosphate, disodium salt, hexahydrate ($C_6H_4NNa_2O_6P \cdot 6H_2O$), $M_r = 371.14$
3. Magnesium acetate [$Mg(C_2H_3O_2)_2 \cdot 4H_2O$], $M_r = 214.46$
4. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), $M_r = 287.54$
5. N-(2-Hydroxyethyl)ethylenediamine-N,N′,N′-triacetic acid (HEDTA), trisodium salt ($C_{10}H_{15}N_2Na_3O_7$), $M_r = 344.20$ (anhydrous)
6. Hydrochloric acid (HCl), $M_r = 36.46$, 25 %
7. Hydrochloric acid (HCl), $M_r = 36.46$, 2 mol/L
8. Sodium chloride (NaCl), $M_r = 58.44$

Table 2 Measurement conditions for the measurement of ALP.

Temperature	37.0 °C ± 0.1 °C[a]
Wavelength	405 nm ± 1 nm[a]
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm[a]
Incubation time	60 s
Delay time	90 s
Measurement interval	120 s
Readings (measurement points)	≥ 6

[a]Maximum allowable expanded uncertainty (95 % probability).

Note: AMP may contain inhibitors for ALP. The manufacturers should declare that the buffer substance is suitable for the investigation of ALP.

Note: The content of water in commercially available HEDTA differs. The percentage of water in the used lot shall be documented in a certificate of analysis of the manufacturers and shall be taken into account by the preparation of the metal ion buffer.

Note: The NPP reagent must meet the following criteria (declared in manufacturers' certificate of analysis or confirmed by investigations of the user):

- Enzymatic conversion of NPP to 4-nitrophenol should result in a hydrolysis of >0.98,
- The molar absorption coefficient of NPP at 311 nm in sodium hydroxide, 10 mmol/L at 25 °C, should be 986.7 $m^2/mol \pm 7.6$ m^2/mol,
- The mol fraction of 4-nitrophenol to NPP must be <0.0003, and
- The mol fraction of inorganic phosphate to NPP must be <0.01.

Detailed procedures for checking these conditions are given by Bowers et al. (14).

Reagents of the highest purity must be used. If a chemical is suspected of containing impurities affecting the catalytic activity of the analyte further investigations must be performed: e.g., comparisons with products from different manufacturers and different lots.

It is recommended to use reagents which have already been tested and approved in comparisons.

Charts for the adjustment and control of pH

Procedure for the adjustment of pH at temperatures diverging from 37 °C

Both the thermometer and the pH electrode are suspended in the mixed solution simultaneously. The stirred solution is then titrated to the pH listed in the chart for the actually measured temperature (Table 3). The speed of agitation should be the same during the calibration and the control of the pH-meter and the adjustment of the pH of the reagent solutions. The pH electrode should be positioned in the center of the stirred solution.

The possibility that the temperature can change during the titration must be taken into account. For this reason, the temperature in the proximity of the target value should be controlled again and the target pH has to be corrected if necessary. The same applies for the adjustment of the temperature compensation of the pH meter.

Preparation of solutions

The given mass of the compounds for the preparation of solutions refers to 100 % content. If the content of the reagent chemical employed is less (e.g., *yz* %), the amount equivalent to the given mass is calculated by the use of a factor:

$$F_{content} = 100/yz$$

Highly purified water shall be used for the preparation of the reagent solutions. Guidelines describing the preparation and testing of reagent water are published elsewhere (15).

The expanded ($k=2$) combined uncertainty (normally distributed) of each weighing procedure (including the uncertainty of the purity of the substance) shall be ≤ 1.5 %.

Solution 1

0.878 g (25.50 mmol/L) HEDTA; 0.367 g (12.75 mmol/L) zinc sulfate; 0.547 g (25.50 mmol/L) magnesium acetate.

- Dissolve the HEDTA in approximately 70 mL water.
- Add the zinc sulfate to the solution.

Table 3 Dependence of the pH of the reaction solution upon temperature.

Temp, °C	pH	Temp, °C	pH	Temp, °C	pH
15.00	10.906	23.50	10.619	32.00	10.352
15.25	10.897	23.75	10.611	32.25	10.344
15.50	10.889	24.00	10.603	32.50	10.337
15.75	10.880	24.25	10.595	32.75	10.329
16.00	10.871	24.50	10.587	33.00	10.321
16.25	10.862	24.75	10.579	33.25	10.314
16.50	10.854	25.00	10.571	33.50	10.306
16.75	10.845	25.25	10.563	33.75	10.298
17.00	10.836	25.50	10.555	34.00	10.291
17.25	10.828	25.75	10.547	34.25	10.283
17.50	10.819	26.00	10.539	34.50	10.276
17.75	10.811	26.25	10.531	34.75	10.268
18.00	10.802	26.50	10.523	35.00	10.260
18.25	10.794	26.75	10.515	35.25	10.253
18.50	10.785	27.00	10.507	35.50	10.245
18.75	10.777	27.25	10.500	35.75	10.238
19.00	10.768	27.50	10.492	36.00	10.230
19.25	10.760	27.75	10.484	36.25	10.223
19.50	10.751	28.00	10.476	36.50	10.215
19.75	10.743	28.25	10.468	36.75	10.208
20.00	10.735	28.50	10.460	37.00	10.200
20.25	10.726	28.75	10.453	37.25	10.192
20.50	10.718	29.00	10.445	37.50	10.185
20.75	10.710	29.25	10.437	37.75	10.177
21.00	10.701	29.50	10.429	38.00	10.170
21.25	10.693	29.75	10.421	38.25	10.162
21.50	10.685	30.00	10.414	38.50	10.155
21.75	10.677	30.25	10.406	38.75	10.147
22.00	10.668	30.50	10.398	39.00	10.140
22.25	10.660	30.75	10.390	39.25	10.132
22.50	10.652	31.00	10.383	39.50	10.125
22.75	10.644	31.25	10.375	39.75	10.117
23.00	10.636	31.50	10.367	40.00	10.110
23.25	10.628	31.75	10.360		

- Add the magnesium acetate only when the zinc sulfate is completely dissolved.
- Dissolve the magnesium acetate in the solution.
- Transfer to a 100 mL volumetric flask.
- Equilibrate volumetric flask and water to 20 °C.
- Fill water up to the calibration mark of the volumetric flask (20 °C).

Stability at 2–8 °C: 3 months.

Reaction solution

8.52 g (956.3 mmol/L) 2-Amino-2-methyl-1-propanol.

- Dissolve in approximately 70 mL water.
- Adjust pH (37 °C) 10.3 – pH (37 °C) 10.5 with 25 % hydrochloric acid.
- Add 10 mL solution 1.
- Adjust pH (37 °C) 10.2 with hydrochloric acid (2 mol/L).
- Transfer to a 100 mL volumetric flask.
- Equilibrate volumetric flask and water to 20 °C.
- Fill water up to the calibration mark of the volumetric flask (20 °C).

Stability at 2–8 °C: 3 months.

Start reagent solution

0.757 g (81.6 mmol/L) 4-Nitrophenyl phosphate, disodium salt, hexahydrate.

- Dissolve in approximately 15 mL water.
- Transfer to a 25 mL volumetric flask.
- Equilibrate volumetric flask and water to 20 °C.
- Fill water up to the calibration mark of the volumetric flask (20 °C).

Stability at 2–8 °C: 1 week.

Measurement procedure

Degas carefully the amount of reaction solution required for the experiment. The degassing can be performed by using a vacuum oven with a temperature set at 35 °C or warming the solution up to approximately 35 °C following by storage of it for 1 h in a vacuum desiccator while stirring with a magnetic stirrer. If the degassed solution is not used within the day, degas it again just before use.

Equilibrate only an adequate volume of start reagent solution at 37 °C in preparation for the measurement procedure. The remaining volume of the start reagent solution should be stored at 2–8 °C.

Pipette the volumes as listed in Table 4 one after another into the cuvette.

Reagent blank rate

To determine the reagent blank rate, the specimen is replaced by 9 g/L (154 mmol/L) sodium chloride solution. The measurement procedure is then carried out as described above.

The reagent blank shows a non-linear kinetic. The acceptable blank rate is positive and $<3.0\times10^{-5}$ s^{-1} (<0.0018 min^{-1}).

Note: Due to the non-linearity of the kinetic, the reagent blanks have a lower reproducibility. It is advisable to perform at least three replicates and use the mean for further calculations.

Sample blank rate

For the determination of the sample blank rate, the start reagent solution is replaced by 9 g/L (154 mmol/L) sodium chloride solution. The measurement procedure is then carried out as described above.

Note: The sample blank rate is determined and documented, but not taken into account for calculation of the catalytic concentration of ALP in control sera and calibrators. In case of the value of the sample blank rate exceeding 1 % of total ALP, a warning that the respective material is not appropriate for calibration should be issued.

Note: The reagent blank rate for the sample blank rate is determined by replacing the start reagent solution **and** the sample by 9 g/L (154 mmol/L) sodium chloride solution.

Note: Effects of the matrix of the sample on the indicator reaction have not been considered due to the omission of NPP from the reaction mixture.

Upper limit of the measurement range

If the change of absorbance exceeds 0.0042 s^{-1} (0.25 min^{-1}) in the measurement interval, an analytical portion of the sample must be diluted with 9 g/L (154 mmol/L) sodium chloride solution and the measurement procedure must be repeated with the diluted specimen (16). The obtained value must then be multiplied by the corresponding factor of the dilution.

Note: The following rules for the dilution of samples are recommended for improved standardization.

The sample shall be diluted if the interval of the combined expanded uncertainty and the upper limit of the measurement range are overlapping. In this case pre-dilution of 10 volume parts of the sample with one volume part of 9 g/L (154 mmol/L) sodium chloride solution is recommended.

Adequate dilution shall be performed if the interval of the combined expanded uncertainty is completely located above

Table 4 Analytical system for the measurement of ALP.

2.000 mL reaction solution
Equilibrate to 37.0 °C
0.050 mL sample
Mix thoroughly and incubate for 60 s. At the end of the incubation time, the temperature of the solution in the cuvette shall have reached 37.0 °C
0.500 mL start reagent solution
Mix thoroughly, wait 90 s and monitor time and absorbance for an additional 120 s

the upper limit of the measurement range. The change of absorbance of the reaction mixture containing the pre-diluted sample shall be in a range from 0.0034 s^{-1} (0.20 min^{-1}) to 0.0038 s^{-1} (0.23 min^{-1})

Sources of error

- The temporal conversion rate is not linear for all samples. The non-linearity depends on the matrix of the material and the catalytic concentration of ALP. Deviations from the described delay time and measurement time can lead to non-commutability of the material and changed method.
- The optimum pH in some control materials and calibrators differs from the described pH of the reference procedure. This leads to an increased sensitivity to the uncertainty of the pH adjustment.
- Absorbed carbon dioxide from the air changes the pH of the reaction solution. Therefore, the container of reaction solution shall be closed tightly. The pH in solution 1 shall be controlled at least weekly.
- The catalytic concentration of ALP in some control materials increases during the storage. Such a material is not suitable as a calibrator or as control material.
- A thorough mixing of the final complete reaction mixture is necessary due to the high buffer concentration. Otherwise the formation of streaks leads to a poor precision of the measurement results.

Calculation

The temporal change of absorbance (s^{-1}) is calculated with analysis of regression (method of the least squares). After subtraction of the reagent blank rate the corrected change of absorbance is multiplied with the factor, $F = 2729$ (measurement at 405 nm, $\varepsilon_{405} = 1869$ m^2/mol).

Note: The use of the molar absorption coefficient $\varepsilon_{405} = 1869$ m^2/mol is recommended by IFCC and IRMM.

The catalytic concentration of ALP is calculated in μkat/L. $\Delta A/\Delta t_{ALP}$ is the change of absorbance after correction of the reagent blank rate (in s^{-1}); b_{ALP} is the catalytic concentration of ALP (μkat/L); and $b_{ALP} = 2729\ \Delta A/\Delta t_{ALP}$. The catalytic concentration in μkat/L can be converted to U/L by multiplication by the factor $f = 60$.

Preliminary reference values

- Females (18–49 years): 0.55–1.64 μkat/L; 33–98 U/L.
- Males (≥20 years): 0.72–1.92 μkat/L; 43–115 U/L.

Details about the definition of the preliminary reference values are given in Appendix II.

Appendix I

Changes in the IFCC reference procedure for measurements at 37 °C compared with the reference method for measurements at 30 °C as described in the original IFCC document

The primary reference procedure is derived from the draft of the IFCC reference method (2, 3), which provides optimized conditions for the measurement of catalytic concentration of ALP at 30 °C.

The measurement temperature of 37 °C instead of 30 °C requires only minor changes of certain measurement parameters to retain the optimum measurement conditions. The modifications are listed and commented on in this appendix. Furthermore, if in comparison to the 30 °C reference method a more accurate specification has become necessary for improving the standardization of the measurements, it is also described here in Table 5.

Appendix II

Determination of preliminary reference intervals

Sample collection was performed in four different European centres: two sites in Milan (Italy) (Ospedale L. Sacco and Ospedale S. Raffaele), one in Nancy (France) (Centre Medicine Preventive) and one in Bursa (Turkey) (Uludag University Medical School). The serum was collected using a BD Vacutainer system (BD Becton, Dickinson and Company) into plastic tubes with serum separator (SST™ II). The samples were allowed to clot at room temperature and centrifuged (2000 *g*) within 1 h. Serum was separated and aliquoted within 4 h from blood collection. The aliquots were frozen at –80 °C. The frozen aliquots were sent in dry ice to the reference laboratory in Hannover where all the analyses were performed. The reference analyses were performed on a Konelab 30i instrument (Thermo Fisher Scientific). The reagents were self-prepared according to the prescriptions in this document. The measurement parameters and the measurement design were configured as closely related as technically feasible. The calibration was performed by use of a set of pooled sera with assigned target values for ALP obtained by measurements using the manually performed primary IFCC reference measurement procedure.

Subjects in Italy and Turkey were specifically enrolled for the reference interval experiment and they gave informed consent and replied to an ad hoc questionnaire; the samples from France were left over samples from those collected for a health screening program. All of the samples were obtained from individuals in the fasting state. In addition to ALP, the following tests were performed locally in all the samples: aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), glucose and creatinine; in addition, total calcium and inorganic phosphate were measured in the Hannover reference laboratory.

The ages of the enrolled subjects spanned from 5 to 87 years, but the number of subjects in the younger and older age ranges was insufficient to provide reliable reference

Table 5 Comparison of the IFCC methods for the measurement temperatures of 30 °C and 37 °C.

37 °C IFCC reference procedure	30 °C reference method	Comment
Specimen of investigation		
Calibration material, control specimens and human sera	Human sera	The reference procedure will be used primarily for the measurement of control samples and calibration materials.
Uncertainty of the measurement temperature adjustment		
Target uncertainty ≤0.1 °C (k=2)	Bias: less ±0.05 °C Imprecision: less ±0.1 °C	High quality spectro-photometer with devices for temperature adjustment and control provide an uncertainty (k=2) of the temperature ≤0.1 °C.
Incubation time		
60 s	No incubation time	60 s are necessary for mixing and closing the lid of the spectrometer. Loss of temperature can be compensated in this time.
Delay time		
90 s	No information about the delay time	The reagent blank rate is non-linear after the addition of the start reagent solution. The non-linearity decreases with increasing delay time.
Measurement time		
120 s	Up to 300 s	The reaction rate is not linear and the non-linearity proportionally increases with the time and the catalytic concentration of ALP. Shorter measurement time implies less non-linearity.
Concentration of AMP in the final complete reaction mixture		
750 mmol/L	350 mmol/L	750 mmol/L AMP is the optimum buffer concentration. The pH value of the buffer solution decreases faster with lower buffer concentrations. The buffer solution is more stable with the increased AMP concentration.
Start of the reaction		
Solution R 2000 µL Serum 50 µL Start solution 500 µL	Solution R 2500 µL Serum 50 µL	The reaction is started with serum in the 30 °C method and with substrate in the 37 °C procedure.
Temperature of the start reagent solution before use		
Start with substrate: the start reagent solution should be 37 °C before use	Start with serum: no temperature equilibration of the serum is described	The use of start reagent solution with ambient temperature decreases the temperature in the cuvette.
Collection of data		
Number of readings ≥6	Monitoring of the increase in absorbance	Modern spectrophotometers employ digital data processing. A number of readings ≥6 should ensure a sufficient precision of the measurement results. Devices for a continuous monitoring are no longer in use.
Determination of the slope (time vs. absorbance)		
Regression analysis of the method of least squares	No information	A well-defined statistical method is necessary to ensure the reproducibility of the calculation of the slope.
Reference values		
See Table 7	No reference values were given	The reference values for women and men were investigated separately.
Unit of catalytic enzymatic concentration		
µkat/L and U/L	µkat/L	U/L is commonly used in clinical laboratory, but µkat/L is based on the SI system.
Sample blank rate		
Determined, but not taken into account	Subtraction	Usually, sample blank rates are not subtracted in routine procedures. Therefore, assigned values in calibrators and control materials are only useful for routine methods, if they include the sample blank rate value.

intervals. Thus, results are presented only for adult pre-menopausal females and adult males. The origin of the participants and their age group distributions are shown in Table 6.

The population sizes from the different sites were similar, but the Turkish group had a larger number of young subjects.

To exclude the possibility that any of the reference individuals were in late puberty, only individuals who were 18 years or older if females and 20 years or older if males were included in the calculation. The decrease in ALP catalytic activity to typical adult ranges is known to differ from

Table 6 Age and origin of the enrolled subjects.

	Age, years	Italy (Milan)	France (Nancy)	Turkey (Bursa)	Total
Females	18–30	32	32	56	120
	31–40	19	21	28	68
	41–49	34	27	8	69
	Total	85	80	92	257
Males	20–30	14	19	56	89
	31–40	20	25	31	76
	41–50	30	19	8	57
	51–60	13	18	0	31
	>60	7	6	0	13
	Total	84	87	95	266

Table 7 ALP reference intervals.

	Females, age 18–49 years	Males, age ≥20 years
Number of subjects	257	266
2.5° percentile (90 % CI)	0.55 μkat/L (0.48–0.59)	0.72 μkat/L (0.65–0.76)
	33 U/L (28.7–35.4)	43 U/L (39.2–45.8)
97.5° percentile (90 % CI)	1.64 μkat/L (1.50–1.73)	1.92 μkat/L (1.84–2.03)
	98 U/L (89.8–103.6)	115 U/L (110.1–121.6)

subject to subject and occurs on average 2 years earlier in females than in males.

Moreover, as for females a progressive increase of both lower and upper reference limit following the menopause is described (17), we excluded females older than 49 years. As for males no age-related increase in ALP has been described, all the data, including those from elderly people, were included in the calculation.

Exclusion criteria

Participants were excluded from the study if suffering for diabetes (glucose >7.0 mmol/L), renal impairment [creatinine >115 μmol/L if females, 133 μmol/L if males], liver damage (AST, ALT or GGT >100 U/L), total calcium and inorganic phosphate outside the reference intervals (2.15–2.60 mmol/L and 0.80–1.50 mmol/L, respectively).

ALP results

The results of adult males and adult pre-menopausal females were compared using the approach proposed by Lahti et al. (18, 19) and partitioning was suggested, so the two groups were considered separately.

Results for women from the three collection sites were compared using the Lahti et al. (18, 19) statistical method and the Turkish group was found to be significantly different from the French group, whereas difference with the Italian group was only marginal; the Italian and French groups were significantly different only at the lower reference limit. Hence, statistically the three groups were different; however, because the Turkish group was significantly younger and the lower reference limit is clinically less relevant, all the data were merged. The differences among the results from men across the three sites were less pronounced than those of the women. The application of the Lahti algorithm indicated only marginal differences.

The reference limits, calculated with the non-parametric approach, are reported in Table 7.

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